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Vascular E-selectin expression correlates with CD8 lymphocyte infiltration and improved outcome in Merkel cell carcinoma

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Abstract

Merkel cell carcinoma (MCC) is an aggressive, polyomavirus-linked skin cancer. While CD8 lymphocyte infiltration into the tumor is strongly correlated with improved survival, these cells are absent or sparse in most MCCs. We investigated whether specific mechanisms of T-cell migration may be commonly disrupted in MCC tumors with poor CD8 lymphocyte infiltration. Intratumoral vascular E-selectin, critical for T-cell entry into skin, was downregulated in the majority (52%) of MCCs (n=56), and its loss was associated with poor intratumoral CD8 lymphocyte infiltration (p<0.05; n=45). Importantly, survival was improved in MCC patients whose tumors had higher vascular E-selectin expression (p<0.05). Local nitric oxide (NO) production is one mechanism of E-selectin downregulation and it can be tracked by quantifying nitrotyrosine, a stable biomarker of NO-induced reactive nitrogen species (RNS). Indeed, increasing levels of nitrotyrosine within MCC tumors were associated with low E-selectin expression (p<0.05; n=45) and decreased CD8 lymphocyte infiltration (p<0.05, n=45). These data suggest that one mechanism of immune evasion in MCC may be restriction of T cell entry into the tumor. Existing therapeutic agents that modulate E-selectin expression and/or RNS generation may restore T cell entry and could potentially synergize with other immune-stimulating therapies.

Keywords

Merkel cell carcinoma; T cell immune evasion; E-selectin; Nitrotyrosine

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Introduction

Merkel cell carcinoma (MCC) is an increasingly common neuroendocrine skin cancer that is at least twice as likely to be lethal as melanoma (Lemos et al, 2010). Although surgery and/or radiation therapy may be curative for patients with localized MCC in the absence of distant metastases, relapses are common and often incurable, with no disease-specific therapies available. Investigation of mechanisms involved in MCC pathogenesis and progression could offer rational targets for future therapies.

The cellular immune response against MCC is particularly relevant in light of the recently discovered causal link between this cancer and the Merkel cell polyomavirus (MCPyV) (Feng et al, 2008), as well as the increased MCC incidence among immune suppressed individuals with human immunodeficiency virus, chronic lymphocytic leukemia, or solid organ transplantation (Penn, 1999; Engels et al, 2002; Heath et al, 2008). Indeed, MCPyV oncoproteins that are persistently expressed in MCC tumors have recently been shown to be targets for CD8 and CD4 T cells (Iyer et al, 2011). Furthermore, several studies suggest that CD8 and CD3 lymphocyte infiltration into MCC tumors is strongly linked to survival (Paulson et al, 2011; Sihto et al, 2012). However, this advantageous robust lymphocytic infiltration into MCC tumors is only present in approximately ~20% of patients (Paulson et al, 2011). Thus, we hypothesized that the inability of the immune response to control MCC may in part be due to blockade of lymphocyte entry into MCC tumors.

One mechanism of T cell exclusion from tumors is downregulation of adhesion molecules on tumor vasculature or on lymphocytes, thereby blocking recruitment of T cells from blood vessels. In skin, expression of endothelial E-selectin adhesion molecule is the earliest step of tethering, rolling, and emigration of cutaneous lymphocyte antigen (CLA)-positive T cells from blood vessels to sites of inflammation (Kupper and Fuhlbrigge, 2004) and cancer (Clark et al, 2008). Indeed, human squamous cell carcinomas (SCCs) have been shown to evade the immune response by downregulating E-selectin on tumor vasculature (Clark et al, 2008). A recent report suggests that E-selectin expression in SCC is downregulated by nitric oxide (NO) produced by tumor-associated myeloid-derived suppressor cells (MDSCs) (Gehad et al, 2012). Protein nitration is a stable biochemical marker of NO production and iNOS/arginase pathway activation and thus can be tracked in archival tissues using an antibody against nitrotyrosine. Indeed, several human cancers, including prostate, colon, and hepatocellular carcinoma (Kasic et al, 2011), show markedly elevated levels of nitrotyrosine, which are associated with a lack of functional tumor infiltrating lymphocytes (Bronte et al, 2005; Nagaraj et al, 2007). We therefore investigated the role of nitrotyrosine and its association with E-selectin downregulation in and CD8 lymphocyte exclusion from MCC tumors.

In this study, we found that increased numbers of E-selectin-positive vessels in the tumor are associated with greater intratumoral CD8 lymphocyte infiltration and improved MCC-specific survival. The downregulation of E-selectin may be a consequence of the high levels of nitrotyrosine expression in MCC tumors. These findings have mechanistic and potential therapeutic implications for MCC.

Results

The fraction of E-selectin-positive blood vessels is decreased in the majority of MCC tumors

Vascular E-selectin is critical for the entry of CLA-positive T cells into the skin under both normal and inflamed conditions (Kupper and Fuhlbrigge, 2004). To determine the presence of E-selectin in MCC tumor vasculature, we stained serial sections of MCC tumors with

antibodies to E-selectin and CD31. A total of 56 MCC tumors from 55 patients were analyzed. Vascular structures, as identified by staining for CD31, were apparent both within the tumor and in the adjacent peritumoral areas (Fig. 1). Among the 56 tumors, the mean number of vessels was similar in intratumoral (35 ± 19 CD31-positive vessels per 200x magnification field) and peritumoral areas (44 ± 24). Strikingly however, when tumors were compared for the fraction of E-selectin-positive vessels within versus outside the tumor, there was a four-fold decrease in the proportion of E-selectin-positive vessels within the tumor as compared to in the tumor periphery ($p < 0.05$; representative tumor seen in Fig. 1B). Further analyses were carried out after stratifying intratumoral or peritumoral areas into low, moderate or high bins ($<1\%$, $1-5\%$ and $>5\%$ of vessels being E-selectin-positive, respectively; Fig. 2A). Among 56 MCC tumors, the fraction of E-selectin-positive vessels inside the tumor was often low (52% of MCCs) as compared to the fraction of E-selectin positive vessels in peritumoral areas (29% of MCCs; $p < 0.05$). In contrast, intratumoral areas of MCCs were less likely to have a high fraction of E-selectin-positive vessels as compared to peritumoral areas (14% vs 32%, respectively; $p < 0.05$; Fig. 2A). Next, to investigate if there was a correlation between E-selectin expression and MCC-specific survival, we compared the fraction of intratumoral E-selectin-positive vessels among MCC patients. We observed a significant trend towards improved survival among patients with tumors expressing a higher fraction of E-selectin-positive vessels within the tumor vasculature ($p < 0.05$ by logrank test for trend; Fig. 2B). There were no significant associations between E-selectin and stage, gender, age or lesion type.

Intratumoral CD8 infiltration is correlated with E-selectin-positive vessels

To analyze the relationship of T-cell infiltration with vascular E-selectin patterns described above, we stained serial sections of 56 MCC tumor specimens from 55 patients for CD8 and the indicated vascular markers (Fig. 1). Tumor CD8 lymphocyte infiltration patterns were categorized as previously described into 6 bins of density in intratumoral and peritumoral sites and subsequently into three infiltration patterns (brisk, sparse or stalled) (Paulson et al, 2011). Of 56 tumors, 25% had a brisk CD8 infiltrate (intratumoral CD8 score of 3 to 5), while 75% had a sparse CD8 infiltrate (intratumoral score of 0 to 2). Among 34 tumors with no or very low CD8 intratumoral infiltrate (intratumoral score of 0 or 1), 41% exhibited a prominent stalled phenotype with high numbers of peritumoral CD8s (peritumoral score 3–5) accumulating within the tissue immediately adjacent to the tumors (representative example seen in Fig. 1B). Among all analyzed tumors, an increasing fraction of intratumoral E-selectin-positive vessels was associated with an increasing intratumoral CD8 lymphocyte score ($p < 0.05$; Fig. 3A). Tumors with a high fraction of E-selectin-positive vessels had a median CD8 lymphocyte score of 3.5, with CD8 scores ≥ 3 previously reported to be associated with excellent MCC-specific survival in a large cohort study (Paulson et al, 2011). In contrast, tumors with a low fraction of E-selectin-positive vessels had a median CD8 lymphocyte score of zero.

In order to explain the distinct CD8 infiltration patterns into MCC tumors (brisk, sparse or stalled as described above) we compared the relative E-selectin-positive fractions between intratumoral and peritumoral vessels. Among tumors with sparse or stalled CD8 infiltrates, the intratumoral E-selectin-positive fraction was selectively decreased as compared to the peritumoral E-selectin fraction ($p < 0.01$; Fig. 3B). In contrast, among tumors with robust numbers of CD8 lymphocytes in the tumor and surrounding stroma (brisk infiltrate), E-selectin positivity was preserved among both intratumoral and peritumoral vessels. Altogether, these studies suggest that the restriction for CD8 lymphocyte entry into some tumors may be mechanistically linked to the low E-selectin-positive proportion of tumor vessels.

Expression of the skin homing receptor CLA is retained on MCC-targeting lymphocytes

Because of its key role in facilitating lymphocyte adhesion to E-selectin and entry into the skin, we determined whether CLA was expressed on lymphocytes in and around MCC tumors. MCC tumor sections were co-stained with CLA and CD8 and co-localization of these proteins was quantified as a percent of total CD8 lymphocytes by immunofluorescence analysis (Fig. 4A). Of 20 tumors, 80% had CLA/CD8 co-expression that was moderate (n=9, defined as 5–50% CLA-positive CD8 cells) or high (n=7; 50% CLA-positive CD8 cells) (Fig. 4A). CD8 T cells from blood had similar levels of CLA expression in MCC patients and control subjects, suggesting no global dysregulation of CLA expression (Fig. 4B). In a small cohort of MCC patients in which it was possible to examine MCPyV oncoprotein-specific CD8 T cells from blood, on average, CLA expression was observed in 39% of these virus-specific T cells as compared to 7% and 4% on T cells specific for CMV and EBV respectively ($p<0.05$) (Fig. 4B). The CLA-negative MCPyV-specific T cells may represent populations that have been primed in other non-skin compartments or cells with central memory rather than effector memory function. Overall, these data suggest that MCC-targeting CD8 lymphocytes, especially those in the MCC tumor microenvironment, often express CLA and would thus be capable of binding its E-selectin ligand when expressed on blood vessels.

High nitrotyrosine levels are associated with low E-selectin-positive vessels and poor CD8 lymphocyte infiltration

Recent studies have reported that local production of nitric oxide (NO) leads to downregulation of vascular E-selectin and impairment of T-cell trafficking into tumors (Gehad et al, 2012). Thus, we stained 236 MCC tumors from 181 patients using an anti-nitrotyrosine antibody to evaluate protein nitration (Molon et al, 2011), which is a consequence of local NO-mediated production of reactive nitrogen species (RNS) (Eiserich et al, 1995; Sawa et al, 2000; Radi, 2004; Szabó et al, 2007; Nathan and Ding, 2010). Approximately 43% of MCC tumors (n=101) had moderate or high expression of nitrotyrosine, with only 6% of tumors completely lacking nitrotyrosine staining within the tumor microenvironment (Fig. 5A). Furthermore, increasing levels of nitrotyrosine were associated with lower number of E-selectin positive vessels within MCC tumors ($p<0.05$; Fig. 5B). Higher nitrotyrosine levels were also associated with lower intratumoral CD8 lymphocyte scores ($p<0.05$; n=45; Fig. 5B). These data suggest that metabolic pathways involving NO and RNS production may be one of several mechanisms regulating T cell extravasation into MCC tumors.

Discussion

The cellular immune system is particularly important in controlling Merkel cell carcinoma given that immune dysfunction is associated with increased incidence (Penn, 1999; Engels et al, 2002; Heath et al, 2008) and diminished survival for MCC (Paulson et al, 2012). Sparse lymphocyte infiltration observed in the majority of MCCs suggests that defective T cell entry may play a role in the inability to control this highly immunogenic cancer. Indeed, we report that vascular E-selectin, required for the recruitment of CLA-positive T cells into the skin, is downregulated in the majority of MCCs. Tumors with a higher number of E-selectin-positive vessels are associated with increased intratumoral CD8 lymphocyte infiltration and with improved MCC-specific survival. Furthermore, we provide evidence that metabolic pathways leading to production of nitrotyrosines are associated with E-selectin downregulation and with poor CD8 T cell infiltration into MCC tumors.

Vascular adhesion molecule expression has clinically significant implications in a number of human cancers. E-selectin is typically expressed in a subset of vessels in normal, non-

inflamed skin (Chong et al, 2004). Depending on the cancer type, the presence of E-selectin may be associated with an improved or worsened prognosis. In breast, colon and lung cancers, elevated E-selectin expression on tumor vasculature recruits pro-tumorigenic immune infiltrates and facilitates attachment and transmigration of tumor cells through the endothelium, effectively promoting cancer progression, metastasis and poorer survival (Mann, 2011). In contrast, in other cancers, including squamous cell carcinoma and melanoma, the proportion of E-selectin-positive vessels is markedly decreased and is associated with a lack of protective T cells within tumor nodules (Clark et al, 2008; Gehad et al, 2012; Weishaupt et al, 2007). The known strong association between intratumoral lymphocyte infiltration and improved survival of MCC patients and the predominant absence of protective lymphocytes in most tumors suggested that vascular endothelium might play an important role in MCC tumor immune escape. Thus, we investigated the association between vascular E-selectin expression, lymphocyte infiltration patterns and survival in MCC. This study expands the limited number of reports on E-selectin relevance and its association with survival in skin cancers. In contrast to other cancers, where E-selectin is often reported as a biomarker of metastatic potential and a predictor of worsened outcome, to our knowledge, the link between vascular E-selectin expression and improved survival has not been previously reported. The presence of E-selectin in the tumor vasculature may be particularly important for immunogenic cancers that are targets of cytotoxic immune cells.

There are several known mechanisms that can contribute to cellular immune escape and diminished lymphocyte infiltration. Loss of E-selectin on the tumor vasculature may prevent adequate leukocyte capture and rolling mediated by E-selectin/CLA interactions on T cells that are capable of reaching the tumor periphery. Recent evidence suggests that there is a strong link between vascular E-selectin downregulation and nitric oxide production by myeloid derived suppressor cells in squamous cell carcinomas (Gehad et al, 2012). It is plausible that similar mechanisms of E-selectin regulation are at play in MCC. Indeed, we observed that nitrotyrosine, a surrogate marker of nitric oxide and reactive nitrogen species production, is associated with E-selectin downregulation and deficient CD8 lymphocyte infiltration. Beyond E-selectin downregulation, additional nitrotyrosine-mediated mechanisms of T cell immune evasion include: 1) block of signaling and responsiveness to antigen via TCR/CD3 ζ nitration (Nagaraj et al, 2010) 2) block of TCR/HLA interactions and tumor recognition by TCR/CD8 nitration (Nagaraj et al, 2007), and 3) prevention of T cell migration via nitration of chemokines which renders them dysfunctional (Molon et al, 2011).

Suboptimal clinical outcomes of adoptive T cell therapy for immunogenic cancers may be in part due to lack of T cell recruitment into tumors. Downregulation of vascular E-selectin and tumor protein nitration present obstacles for appropriate tumor entry and activity of therapeutic tumor-targeting T cells. Importantly, studies in a variety of cancers suggest that improved T cell infiltration and function may be achieved by therapeutic modulation of pathways regulating E-selectin (Clark et al, 2008) and protein nitration (Molon et al, 2011). Specifically, E-selectin induction has been observed in vitro with TNF- α and IL-1 cytokines (Wyble et al, 1997), angiostatins (Luo et al, 1998) and topical imiquimod (Clark et al, 2008). Recent studies also showed that inhibitors of nitric oxide synthase activity were effective in both E-selectin upregulation (Gehad et al, 2012) and reversal of nitrotyrosine-associated T cell dysfunction (Bronte et al, 2005; De Santo et al, 2005). Furthermore, drugs that block the generation of reactive nitrogen species can increase tumor-specific CD8 T cell recruitment and reduce tumor growth when given in combination with adoptive immunotherapy in mice (Molon et al, 2011).

This study was limited to the examination of formalin-fixed paraffin-embedded human MCC tissues. Future investigations on fresh or frozen MCC tumors using multi-color immunofluorescence markers may reveal the phenotypic identity of cells that induce protein nitration of MCC tumors. All of our studies were on human tissue, hence reflecting human disease, however, this posed obstacles in determining causality of our observations. Although an animal model would have advantages, existing MCC xenograft models require profoundly immune deficient mice, and thus would not be able to address most of the relevant aspects of the immune response in MCC. It is plausible that future studies in transgenic mouse models that mimic MCC pathogenesis (such as the spontaneous carcinogenesis model induced by sporadic SV40 polyomavirus oncoprotein expression (Cz  h et al, 2010)) may be useful in studying immune responses to molecules that target E-selectin, nitrotyrosine, and elucidating other relevant mechanisms involved in T cell trafficking such as NF-  B regulation of adhesion molecules and chemokines (Liou, 2002). Furthermore, trials in MCC patients using E-selectin upregulating agents as discussed above, may validate the observed associations between E-selectin upregulation, enhanced CD8 lymphocyte infiltration and improved survival. Although we have limited our studies of cutaneous immunosurveillance to investigation of E-selectin, other contributory mechanisms include the recruitment of CCR8⁺ T cells by constitutively expressed CCL1 in the skin (Schaerli et al, 2004) and platelet (P)-selectin mediated cutaneous T cell migration (Kulidjian et al, 2002).

In summary, this study provides insight into immune evasion mechanisms that likely play a role in diminishing lymphocyte entry into MCC tumors. As it is feasible to target these pathways using existing or emerging agents, it may be appropriate to combine such treatment with adoptive T cell therapy to improve migration of T cells into tumors and thereby augment the efficacy of future immune therapy.

Materials and Methods

Tissue and blood samples

This study was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board and conducted according to Declaration of Helsinki principles. Written informed consent was received from participants prior to inclusion in the study. A total of 248 formalin-fixed paraffin-embedded tumors from 192 patients were analyzed (Table 1). Blood samples were collected from MCC patients (n=11) and healthy volunteers (n=10) who were used as control subjects.

Immunohistochemistry and immunofluorescence

Serial tumor sections were stained with hematoxylin and eosin, and with antibodies against E-selectin (Novocastra, clone 16G4, 1:50 dilution), CD31 (Dako, clone JC70A, 1:100 dilution), CD8 (Novocastra, clone 4B11, 1:200 dilution), CLA (BioLegend, clone HECA-452, 1:100 dilution), and nitrotyrosine (Millipore, rabbit polyclonal, 1:250 dilution). The specificity of the nitrotyrosine antibody was validated using colon tissue treated with peroxynitrite as a positive control and degraded peroxynitrite as a negative control (Supplementary Figure S1)(Sawa et al, 2000; Molon et al, 2011). Scoring for all studies was performed by observers who were blinded to all subject characteristics.

Intratumoral and peritumoral E-selectin-positive vessels were scored among 56 MCC tumor specimens from 55 patients using a three-tiered system: absent/Low = <1%, moderate = 1–5%, high >5%, expressed as a percent of CD31-positive vessels in serial sections. The score represented the average fraction of E-selectin-positive vessels in the entire intratumoral or peritumoral areas with at least 8–10 tumor fields scored when possible. To compare

peritumoral and intratumoral areas, the fold-difference (ratio of the percentages of E-selectin-positive vessels) was calculated for each tumor, and then averaged over all tumors. Intraobserver variability was evaluated in a random sample of 13 tumors. Observed agreement was 80% and weighted kappa statistic was 0.55, consistent with fair to good agreement between observers (Fleiss, 1981).

Fifty-six MCC tumors from 55 patients were assessed for CD8 lymphocytes using a previously described scoring system (Paulson et al, 2011). Briefly, intratumoral and peritumoral CD8 infiltrates were scored separately on a 0 to 5 scale with 0 representing no CD8 cells and 5 representing a strong CD8 infiltrate. Approximate numbers of CD8+ cells per mm² were quantified for each 0 to 5 bin (with an average of 0, 90, 306, 508, 675, 732+ CD8s/mm², respectively). Intratumoral CD8 lymphocytes were those that were surrounded by tumor cells and did not have direct contact with stroma. Tumor CD8 lymphocyte infiltration patterns were categorized as sparse (intratumoral CD8 score = 2), brisk (intratumoral CD8 score = 3), or stalled (intratumoral CD8 score = 1 and peritumoral CD8 score = 3).

For dual staining of CLA and CD8 immunofluorescence studies, sections were incubated with anti-CLA as above followed by biotinylated goat-anti-rat (1:50, Jackson ImmunoResearch) and streptavidin AlexaFluor-568 (1:200, Invitrogen). The same sections were stained with anti-CD8 (Dako, 1:50, clone C8/144B) followed by goat-anti-mouse AlexaFluor-647 (1:50, Invitrogen). 4,6-diamidino-2-phenylindole was used for nuclear staining. CLA/CD8 coexpression was quantified as the number of cells with CLA and CD8 co-localization as a percent of total CD8-positive cells. The fraction of CD8 lymphocytes co-expressing CLA was assessed in the whole tissue specimen and was categorized as none/low (<5%), moderate (5–50%), or high (>50%). Sections were captured using ScanScope model FL (Aperio), acquired and analyzed with Spectrum version 11.1.1.764 (Aperio), and confirmed with Definiens Architect XD Tissue Studio IF software version 2 (Definiens).

Three observers assessed nitrotyrosine staining. Tissue microarrays (TMA) of tumor cores were scored using a semiquantitative integrated assessment of intensity and proportion staining and categorized as follows: none, low, moderate or high staining. The median of the observers' scores was calculated. TMA cores contain mostly tumor cells, but both tumor and stroma areas were included in the score.

Flow cytometry analysis

PBMC were thawed from cryopreserved heparinized blood separated with Ficoll/Hypaque. Lymphocytes were incubated with APC-conjugated HLA/peptide tetramers specific for MCPyV (A24/MCPyV.LT-92-101), CMV (A2/CMV.pp65.495-503) or EBV (A2/EBV.BMLF1.280-288) for 30 minutes at 37°C. Fc receptor blocking reagent (Myltenyi Biotech) was then added for 10 min at 4°C. Next, cells were stained with CD3-Qdot605 (Invitrogen, clone 7D6/S4.1), CD8-V500 (BD Biosciences, clone RPA-T8), and CLA-FITC (Biolegend, clone HECA-452) for 30 min at 4°C. Cells were washed and fixed. Events were collected on a FACS Aria II machine (BD Biosciences) and analyzed using FlowJo software (Tree Star). Analysis and gating were carried out on CD3⁺CD8⁺ or CD3⁺CD8⁺Tetramer⁺ T cells from blood of MCC patients or control subjects.

Statistical analysis

Wilcoxon rank-sum test was used to assess significance among categorically ordered groups. Cuzick's nonparametric test for trend (Cuzick, 1985) was used to assess trend across ordered groups. Student's t-test was performed when comparing means among two groups. Fisher's exact test was used to determine associations between two categorical variables.

Kaplan-Meier survival curves of cause-specific survival were generated using preselected E-selectin category cutoffs (low, moderate, high) and statistical significance was determined using logrank test for trend. P-value <0.05 was considered significant. All analyses were performed with Stata software (StataCorp).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CLA	cutaneous lymphocyte antigen
MCC	Merkel cell carcinoma
NO	nitric oxide
RNS	reactive nitrogen species

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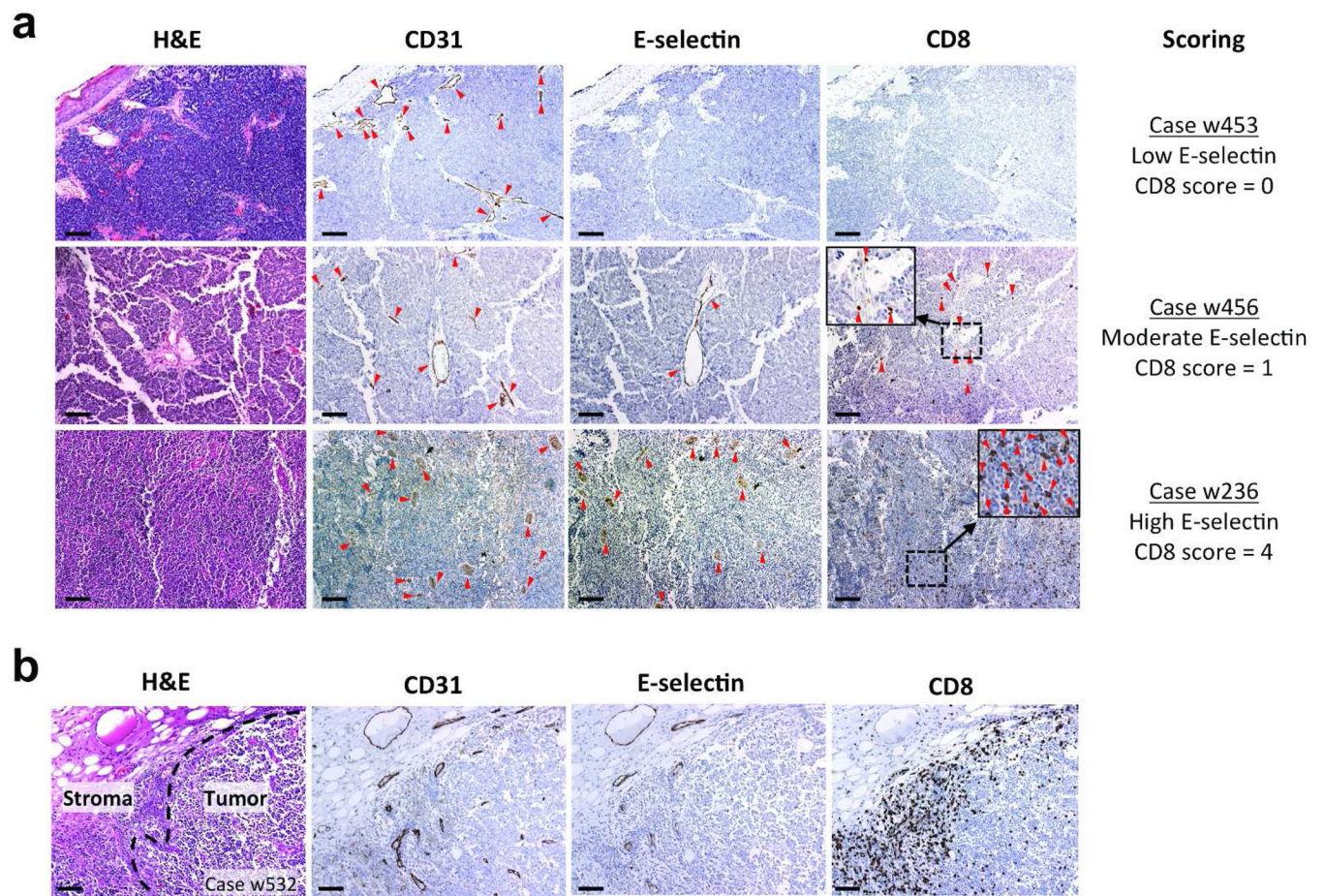


Fig 1. Vascular adhesion molecules and CD8 infiltration on representative serial tumor sections
(a) *Left to right:* Serial sections stained as indicated from 3 patients (cases w453, w456, w236) with the specified intratumoral CD8 and E-selectin scores. Red arrowheads indicate areas of positive staining on immunohistochemistry for the indicated antibody. Scale bar: 100µm. **(b)** *Left to right:* Serial sections from an MCC tumor (case w532) with both stromal and tumor components stained with specified antibodies. Black dashed line indicates junction between tumor and stroma. The sections shown are representative of staining patterns in the stroma and tumor. Scale bar: 100µm.

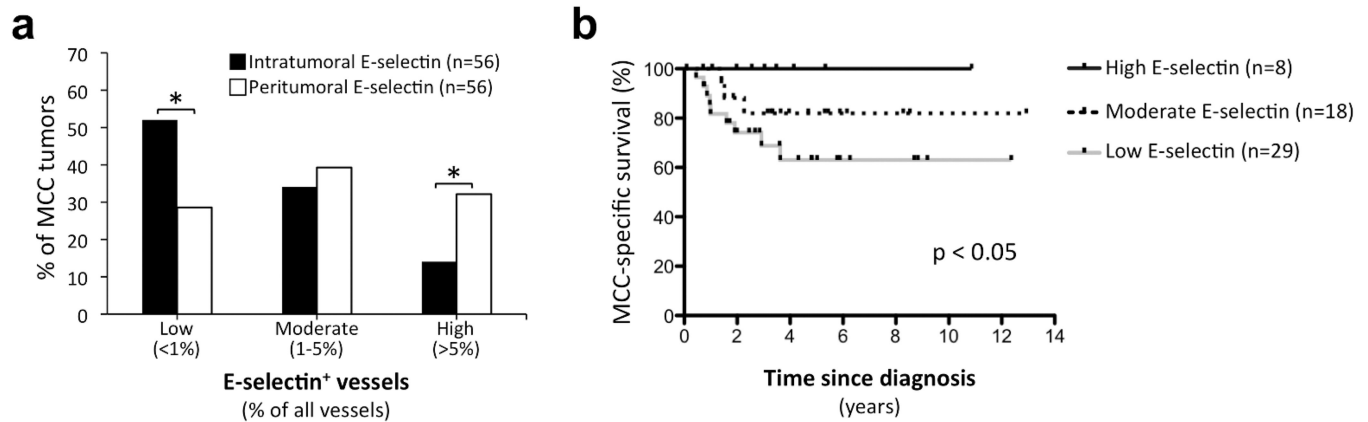


Fig 2. MCC tumors often have decreased E-selectin-positive vessels, which correlate with worse survival

(a) Percent of MCC tumors with low (<1%), moderate (1–5%) or high (>5%) fraction of intratumoral (black bars) or peritumoral (white bars) E-selectin-positive vessels. **p*<0.05, Fisher's exact test. **(b)** Kaplan-Meier curves showing MCC-specific survival of patients with low (n=29), moderate (n=18) or high (n=8) fractions of intratumoral vessels that were E-selectin-positive. P-value determined by logrank test for trend.

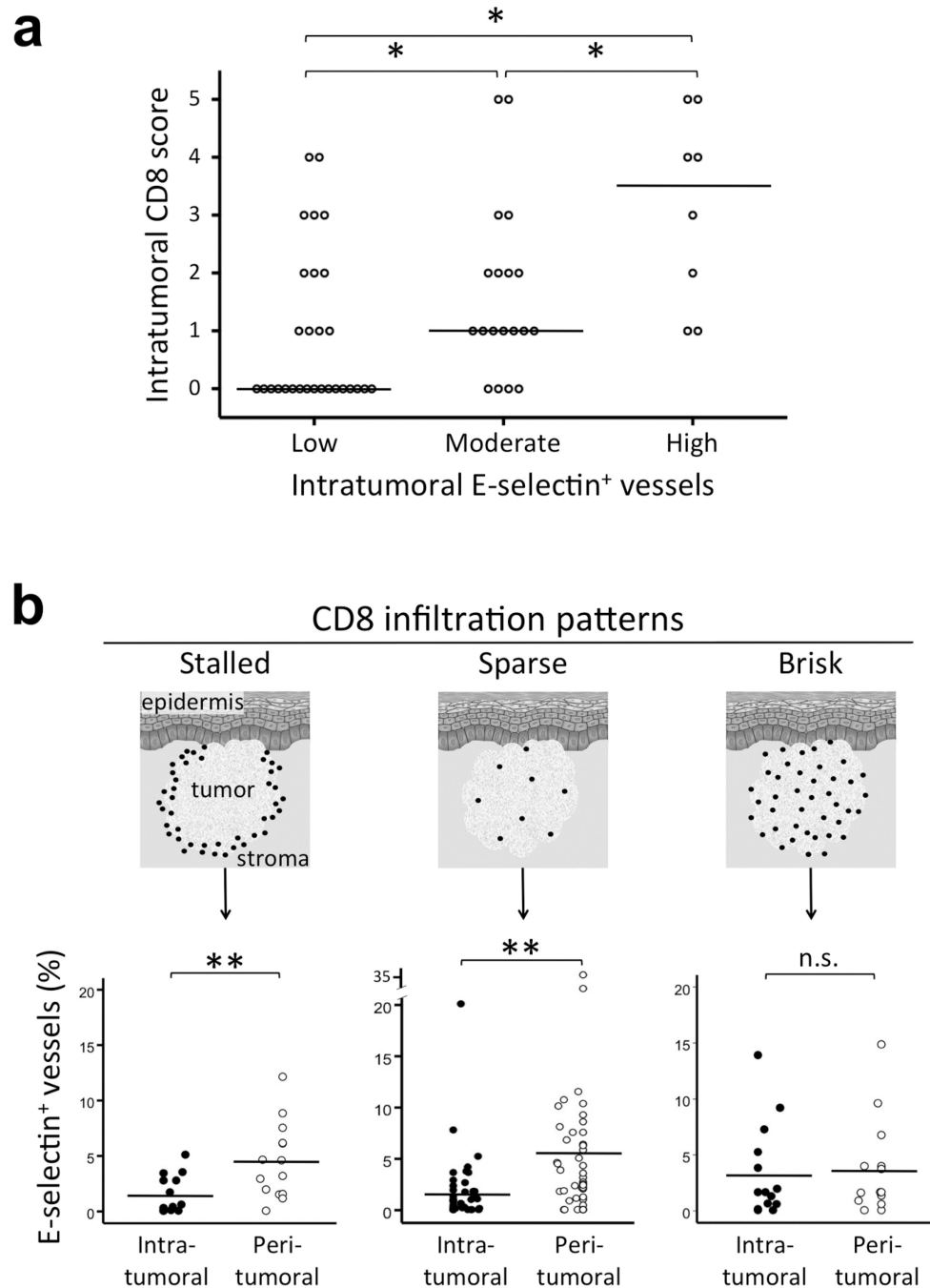
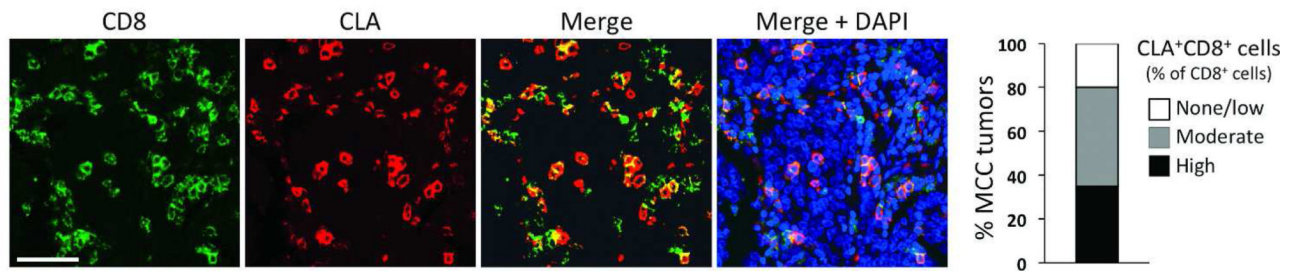


Fig 3. Intratumoral E-selectin is associated with CD8 lymphocytic infiltration into MCC tumors
(a) Correlation between intratumoral E-selectin-positive vessels and CD8 lymphocyte infiltration in 56 MCCs. E-selectin was scored as a percent of all vessels and stratified as low (<1%, n=29), moderate (1–5%, n=19) or high (>5%, n=8). CD8s were scored on a 0–5 scale (Paulson et al, 2011). Black bar indicates median. *p<0.05, Wilcoxon's ranksum test.
(b) Comparison of intratumoral (filled circles) versus peritumoral (empty circles) E-selectin-positive vessels among tumors with CD8 infiltrates characterized as stalled (intratumoral CD8 score 1 and peritumoral CD8 score 3, n=14), sparse (intratumoral CD8 score 2,

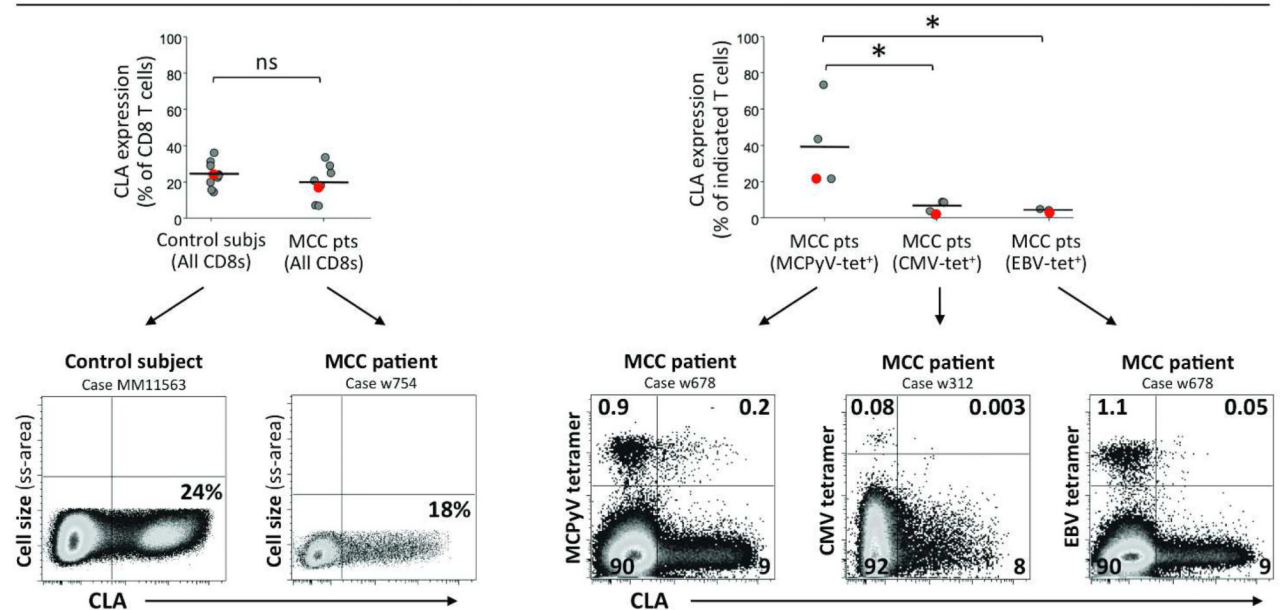
n=42) or brisk (intratumoral CD8 score ≥ 3 , n=14). Black dots in schematic = CD8 lymphocytes. Black bar indicates mean. **p<0.01, Student's t-test.

a

CLA/CD8 co-expression in MCC tumors

**b**

CLA expression in CD8 T cells from blood

**Fig 4. CLA expression on MCC-specific lymphocytes**

(a) CLA/CD8 co-expression as evaluated by immunofluorescence with the indicated stains in a tumor (high CLA/CD8 co-expression; case w588). *Right:* Fraction of MCCs with CLA/CD8 coexpression categorized as none/low ($n=4$, 5% CLA-positive CD8 cells), moderate ($n=9$, 5–50% CLA-positive CD8s) or high ($n=7$; 50% CLA-positive CD8s). Scale bar: 50 μ m (b) CLA expression in blood as evaluated by flow cytometry. *Top left:* Summary data of CLA expression among CD3⁺CD8⁺ cells from control subjects ($n=10$) and MCC patients ($n=8$). *Top right:* CLA expression among CD3⁺CD8⁺Tetramer⁺ cells specific for MCPyV ($n=4$), CMV ($n=4$), and EBV ($n=3$). The red dot on each graph indicates the representative sample selected for flow plot display below. Black bar indicates mean. Tet⁺, tetramer-positive. * $p<0.05$, Wilcoxon's ranksum test.

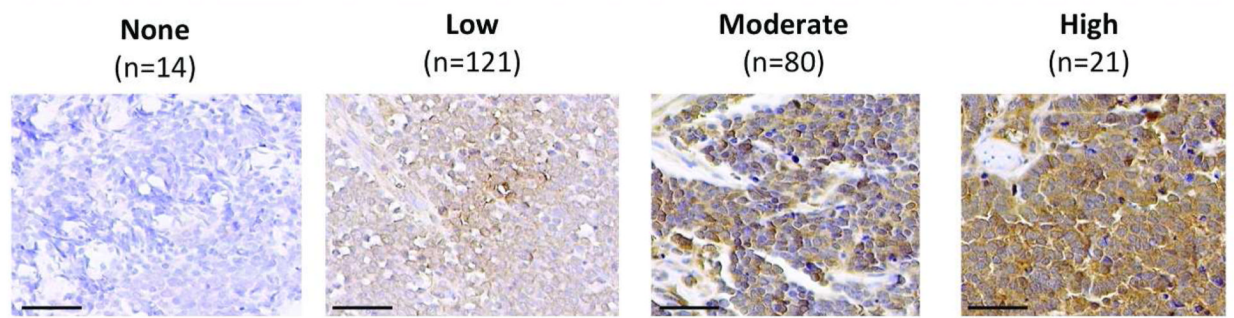
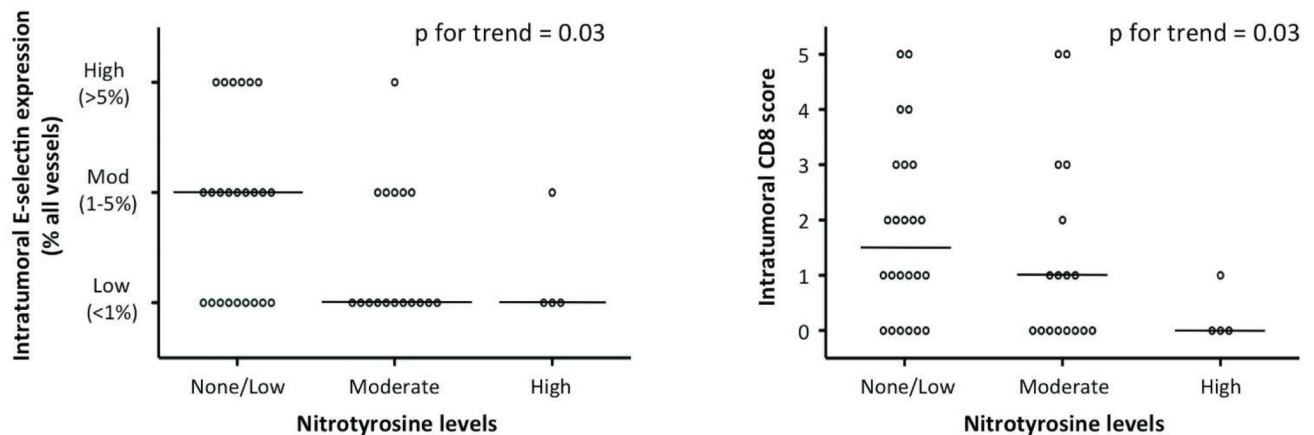
a**Nitrotyrosine expression in MCC tumors (n=236)****b**

Fig 5. High levels of tissue nitrotyrosine are associated with a reduced fraction of E-selectin-positive vessels and poor CD8 lymphocyte infiltration

(a) Representative MCC tumor cores stained for nitrotyrosine (brown). Nitrotyrosine scores took into account both intensity and proportion and were categorized as: none (n=14), low (n=121), moderate (n=80) and high (n=21) expression. Scale bar: 50µm. (b) Correlation between nitrotyrosine levels and intratumoral E-selectin (left) and CD8 (right) scores among 45 MCC tumors. Black bar represents median. P-value determined by Cuzick's nonparametric test for trend.

Table 1

Patient Demographics.

MCC patient and tumor characteristics	Number (%)		
	Subjects	Tumors	Blood
Total number in all studies	196	248	11
E-selectin studies	55	56	--
CD8 studies	55	56	--
CLA studies	31	20	11
Nitrotyrosine studies	181	236	--
Sex			
Male	129 (66%)		
Female	67 (34%)		
Age			
< 65	60 (31%)		
65	136 (69%)		
MCC Stage at presentation			
I (Local ≤2cm)	45 (23%)		
II (Local > 2cm)	29 (15%)		
III (Nodal)	73 (37%)		
IV (Distant metastasis)	20 (10%)		
Unknown	29 (15%)		
Lesion type studied (n=248)			
Primary	154 (62%)		
Regional metastasis/recurrence	58 (23%)		
Distant metastasis	22 (9%)		
Unknown	14 (6%)		

Note: Patients with nodal presentation and unknown primary are represented in the "regional metastasis" lesion type. Due to insufficient data, 29 patients could not be staged and are listed as "unknown". A total of 248 tumors were analyzed from 192 patients in at least one of the studies. Four additional patients donated blood only for the CLA study.